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Calcium effect on the mycelial cell walls of *Botrytis cinerea*

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Abstract

The goal of this study was to determine whether calcium ion, (one of the electrolytes released after plant cell attack), may have a direct effect on fungal growth and chemistry of the fungal cell wall. *B. cinerea* was grown on Richard's solution containing different amounts of $CaCl_2$, and the cell walls were extracted from the mycelium after 7 days of growth. Mineral, neutral and aminosugar, protein and uronic acid contents were determined. At 1 g l^{-1} $CaCl_2$, only the aminosugar content increased. At 2 g l^{-1} $CaCl_2$, neutral sugar synthesis was reduced, whereas the uronic acid content increased. For higher $CaCl_2$ concentrations, the calcium ion content of the cell wall increased, resulting in reduced protein and neutral sugar contents. Meanwhile, the cell wall proportion of the mycelia increased on a dry weight basis due to an increase in uronic acid, Ca, P, Na and neutral sugar contents of the cell wall with increasing $CaCl_2$ in the media. The resulting thickening of the fungal cell wall caused by calcium ion may be an important factor in the host-pathogen relationship.

Keywords: *Botrytis cinerea*; Fungal cell wall; Calcium

1. Introduction

In plants, calcium (Ca) strengthens cell walls by chelating pectic substances (Poovaiah, Glenn & Reddy, 1988), and the resistance of plant tissue to infection by fungi is increased by calcium accumulation in plant cells (Chardonnet & Donèche, 1995; Tobias, Conway, Sams, Gross & Whitaker, 1993). When plant tissues are colonized by a pathogen like *Botrytis cinerea* Pers.: Fr., the cell walls are degraded, leading to a leakage of electrolytes from both the plant cell wall and cytoplasm (Kaile, Pitt & Khun, 1991). The effect of these electrolytes, particularly calcium ion, on the infection process has not been adequately investigated. Only traces of calcium ion are sufficient for the growth of this fungus (Dhingra & Sinclair, 1985), which makes the study more complex.

Calcium ion-containing sprays and postharvest treatments can decrease pre- or postharvest fruit infection by *B.cinerea* (Conway, Sams, Abbott & Bruton, 1991; Elad, Yunis & Volpin, 1993; Kubik, Michalczuk & Malinowski, 1991). We have shown that externally applied calcium ions accumulate in plant tissues, especially in the cell wall (Conway & Sams, 1983; Conway, Gross & Sams, 1987). The resulting Ca^{2+} bridges in the cell wall between pectic acids or between pectic acids and other acidic polysaccharides hindered accessibility to the cell wall of pectolytic enzymes produced by fungal pathogens during decay (Conway, Gross, Boyer & Sams, 1988).

The fungal cell wall of Deuteromycetes like *B.cinerea* is composed of glucans, chitin, proteins, lipids, inorganic ions (Aronson, 1965) and uronic acids (Bartnicki-Garcia, 1968; Blake & Richards, 1970). The detailed mineral composition of fungal hyphae has not been adequately investigated (Bartnicki-Garcia & Nickerson, 1962). As in plants, the fungal cell wall is responsible for cell shape, protection from the environment and mechanical rigidity of the hyphae (Aronson,

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1965). Mycelial growth is promoted by the pressure of the cytoplasmic membrane on the cell wall, apparently not by hormonal stimuli as in plants (Wessels, 1986); (cover case). Mycelial growth, then, depends on the structural and chemical properties of the cell wall. Changes in the composition or the synthesis of cell wall polymers could lead to alterations in fungal morphology and physiology and possibly affect pathogenicity. The hypothesis that Ca^{2+} released from plant cells after enzymatic digestion by a fungal pathogen can interfere with the infection process has been investigated by several authors. Most studies concentrated on the inhibition of the fungal enzymes involved in the digestion of the plant cell wall (Pagel & Heitefuss, 1990; Volpin & Elad, 1991), mycelial viability (Kaile, Pitt & Khun, 1992) and pathogenicity (Chardonnet, 1994).

Our earlier work focused on the mechanism by which exogenously applied Ca^{2+} stabilized the plant cell wall and protected it from cell wall degrading enzymes produced by fungal pathogens (Conway & Sams, 1983; Conway et al., 1987, 1988). In contrast, the objective of this study was to determine the direct effect of Ca^{2+} accumulation on *B.cinerea* by determining if the fungus can absorb Ca^{2+} ions, and if so, the resulting effect on hyphal growth and chemical composition of the mycelial cell wall.

2. Results

On a dry weight basis, no significant difference in growth was observed from 0 to 16 g l^{-1} $CaCl_2$ (Fig. 1). *B. cinerea* can grow at a very low extracellular Ca^{2+} level (the control media contained 3.73 ± 0.54 mg l^{-1} Ca^{2+}) and Ca^{2+} does not stimulate fungal growth. The percent dry wt of the mycelia that consisted of cell wall material increased when the fungus was grown in media containing from 1 through 16 g l^{-1} $CaCl_2$ compared to the control (Table 1). At 8 g l^{-1} $CaCl_2$, the cell walls represented 61% (on a dry wt basis) of the total dry wt of the hyphae. There was, therefore, an actual increase in percent cell wall content in the mycelium as the $CaCl_2$ concentration was increased.

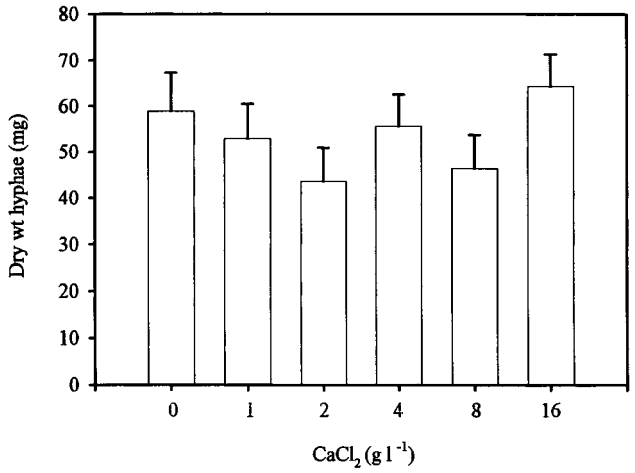

Fig. 1. The effect of calcium on the growth of *Botrytis cinerea* hyphae. Error bars indicate standard error of the means.

Table 1
Calcium chloride effect on the cell wall content of *Botrytis cinerea* hyphae[a]

$CaCl_2$ (g l^{-1})	Cell wall content (% of dry wt hyphae)
0	37.4 d
1	42.8 b
2	39.6 c
4	39.7 c
8	60.9 a
16	56.0 b

[a] Asymptotic normality of the percentage was used to construct a normal-theory procedure for determining confidence limits for the mean percentages, means with the same letter are not significantly different; $P < 0.05$.

Mineral analysis of the hyphae indicated that K^+, P, Mg^{2+}, Na^+ and Fe were the predominant ions (concentration >1 mg mg^{-1} dry wt) in the control hyphae and represented respectively 60, 23.6, 5.6, 5.2 and 3.1% of all the ions measured (Table 2). The minor elements (concentration <1 mg mg^{-1} dry wt) were B (1.2%) and Al (0.5%). Ca^{2+} was one of the least abundant ions and comprised only 0.17% of the total ion content in the control. The presence of Ca^{2+} in the culture medium decreased K^+ accumulation in the hyphae. At 16 g l^{-1} $CaCl_2$, the percentage of K^+ was less than 10% of all the elements measured. P accumulation was higher at 8 and 16 g l^{-1} $CaCl_2$ compared to that at lower $CaCl_2$ concentrations. At 8 g l^{-1} $CaCl_2$ and higher, the concentration of absorbed Ca^{2+} was significantly higher than at lower $CaCl_2$ concentrations, representing more than 50% of the total elements concentration. Al and Mg^{2+} accumulation was significantly reduced at 4 g l^{-1} $CaCl_2$ and 16 g l^{-1} $CaCl_2$ respectively. The only significant difference in Na^+ content of the hyphae was at 2 g l^{-1} $CaCl_2$. While there were seemingly large differences in the B and Fe contents, these differences were not significant.

Similar to the hyphae, Ca^{2+} was one of the least abundant ions in the cell walls (Table 3). K^+ was mainly located in the intracellular compartment and its concentration in the cell walls was below the ICP detection limit (0.5 mg mg^{-1} dry wt cell wall). Na^+ concentration decreased at all $CaCl_2$ concentrations except 8 g l^{-1} and Ca^{2+} and P concentrations increased in the cell walls at 8 and 16 g l^{-1}. At 2 and 4

Table 2
Element accumulation in *Botrytis cinerea* mycelium when grown with increasing $CaCl_2$ concentrations (results expressed in μg mg^{-1} dry wt hyphae)[a]

$CaCl_2$ (g l^{-1})	Al	B	Ca	Fe	K	Mg	Na	P
0	0.27 ab	0.63 ab	0.09 b	1.60 abc	30.97 a	2.84 a	2.70 bc	12.07 b
1	0.34 a	0.75 a	2.94 b	0.44 c	25.07 a	2.15 b	3.98 ab	11.88 b
2	0.34 a	0.75 a	9.53 b	3.03 ab	14.03 bc	1.63 c	4.55 a	12.75 b
4	0.22 abc	0.47 ab	21.90 b	2.27 ab	14.72 b	2.23 b	3.62 ab	17.78 b
8	0.18 bc	0.33 b	53.40 a	3.16 a	11.18 bc	1.28 cd	3.11 b	31.43 a
16	0.16 bc	0.53 ab	73.82 a	2.28 ab	8.17 bc	0.88 de	2.70 bc	33.92 a

[a] Duncan's multiple range test was used for statistical analysis; means within columns with the same letter are not significantly different; $P < 0.05$.

g l^{-1} $CaCl_2$, a decrease in B was observed. An increase in Mg^{2+} contents occurred at 4 g l^{-1} $CaCl_2$. Ca^{2+} ions present in the culture media had no statistically significant effect on the concentrations of Al and Fe in the cell wall. Although there was a large increase in Fe content, it was not statistically significant. Hence, Ca^{2+} ions are one of the least abundant ions in *B. cinerea* in both intracellular and cell wall components. When grown with increasing Ca^{2+} ion concentrations, *B.cinerea* hyphae accumulated Ca^{2+} in both its cytoplasmic components and cell wall, and this accumulation was directly proportional to the amount of Ca^{2+} present in the culture media. There was a statistically significant quadratic relationship between $CaCl_2$ content in the culture media and mycelia Ca^{2+} concentration ($y = -3.96 + 8.34x - 0.214\ x^2$, $R^2 = 0.99$). There was also a significant quadratic relationship between $CaCl_2$ content and cell wall Ca^{2+} concentration ($y = -8.29 + 10.67x - 0.248x^2$, $R^2 = 0.97$).

Ca^{2+} accumulation in the hyphae led to a highly significant decrease in cell wall protein content at 4, 8 and 16 g l^{-1} $CaCl_2$ (Table 3). This decrease (about 30%) may be due to an inhibition of wall protein synthesis. Uronic acid content of *B.cinerea* cell walls was very low (0.86% for the control). This content was significantly increased at 2 g l^{-1} $CaCl_2$ compared to the control, but not at higher $CaCl_2$ concentrations.

To further investigate the effect of Ca^{2+} on the cell wall, the data were expressed on a mycelial dry wt basis (Table 4). The increase in cell wall content of the hyphae observed at 8 g l^{-1} $CaCl_2$ (Table 1) was due to a significant increase in total neutral sugar and uronic acid contents and an accumulation of cations such as Ca^{2+}, P and Na^+. The conclusions previously stated for the Ca^{2+} effect on aminosugar (glucosamine) content were also verified on a mycelial dry wt basis. A slight decrease in protein content was observed at 4 and 8 g l^{-1} $CaCl_2$. In order to estimate the Ca^{2+} effect on the chemical properties of the fungal wall, hydrolysates of cell walls digested with 6 N HCl (a weaker acid than the conc H_2SO_4 used for the determination of total neutral sugars was used because the stronger acid would totally degrade the carbohydrates) were used for neutral sugar determination (Table 5). In the control, the amount of sugar hydrolysed with HCl was ca 80% of the total neutral sugar hydrolysed with

Table 3
The effect of calcium chloride on the chemical composition of *Botrytis cinerea* cell walls (results expressed in μg mg^{-1} dry wt cell wall)[a]

	$CaCl_2$ in culture media (g l^{-1})					
	0	1	2	4	8	16
Total neutral sugars (hydrolysed in H_2SO_4)	580.20 ab	621.43 a	423.26 cd	541.63 abc	502.52 abcd	390.52 d
Aminosugars	154.16 b	225.40 a	183.26 ab	152.56 b	152.37 b	139.22 b
Proteins	28.76 a	26.34 ab	24.74 abc	21.51 cd	19.04 d	19.80 d
Uronic acids	8.64 b	9.20 ab	11.90 a	8.94 ab	9.98 ab	8.87 ab
Na	5.54 a	4.52 b	4.38 b	4.26 b	5.40 a	4.29 b
P	4.39 bc	3.56 c	4.74 bc	19.45 b	38.45 a	52.24 a
Fe	2.58 ab	0.86 b	1.62 ab	3.93 a	4.38 a	4.13 a
B	1.83 a	1.57 ab	1.50 b	1.46 b	1.59 ab	1.53 ab
Al	0.91 a	0.80 a	0.74 a	0.87 a	0.85 a	0.82 a
Mg	0.65 b	0.28 b	0.28 b	1.11 a	0.51 b	0.33 b
Ca	0.21 c	0.75 c	2.53 c	27.41 c	67.90 b	97.50 a
Total	787.87 a	894.71 a	658.95 a	783.13 a	803.00 a	719.25 a

[a] Duncan's multiple range test was used for statistical analysis; means within rows with the same letter are not significantly different; $P < 0.05$.

Table 4
Amount of each cell wall component in *Botrytis cinerea* hyphae (results expressed in mg 100 mg^{-1} dry wt hyphae)[a]

	$CaCl_2$ in culture media (g l^{-1})					
	0	1	2	4	8	16
Total neutral sugars (hydrolysed in H_2SO_4)	21.70 bc	26.60 ab	16.76 c	21.50 bc	30.60 a	21.87 bc
Aminosugars	5.76 b	9.65 a	7.26 ab	6.06 b	6.05 b	7.80 ab
Proteins	1.07 ab	1.13 ab	0.98 bc	0.85 cd	0.75 d	1.11 ab
Uronic acids	0.32 b	0.39 b	0.47 ab	0.35 b	0.61 a	0.50 ab
Na	0.21 bcd	0.19 cde	0.17 de	0.17 e	0.33 a	0.24 b
P	0.16 b	0.15 b	0.19 b	0.77 b	2.34 a	2.92 a
Fe	0.10 bc	0.04 c	0.06 c	0.16 abc	0.27 a	0.23 ab
B	0.07 b	0.07 b	0.06 b	0.06 b	0.10 a	0.09 a
Al	0.03 c	0.03 bc	0.03 c	0.03 bc	0.05 a	0.05 ab
Mg	0.02 bc	0.01 bc	0.01 c	0.04 a	0.03 ab	0.02 bc
Ca	0.01 b	0.03 b	0.10 b	1.09 b	4.13 a	5.45 a
Total	29.51 cd	38.24 abcd	26.06 d	31.13 bed	48.92 bcd	40.20 abc

[a] Duncan's multiple range test was used for statistical analysis; means within rows with the same letter are not significantly different; $P < 0.05$).

conc H_2SO_4. With increasing Ca^{2+} in the culture media, this proportion tended to decrease but no significant difference was found compared to the control. The HCl-hydrolyzed sugars included mannose, glucose and galactose and their respective concentrations were affected by Ca^{2+}. Glucose decreased significantly at 8 g l^{-1} and higher concentrations of $CaCl_2$ as compared to the control, while mannose and galactose increased. Glucans were less susceptible to HCl-hydrolysis when hyphae were grown in the presence of high Ca^{2+} concentration.

3. Discussion

Incubating *B.cinerea* spores in increasing concentrations of $CaCl_2$ (4–26 g l^{-1}) resulted in decreased spore germination and germ-tube growth. This effect was partially reduced by the presence of 5 mM glucose in the medium (Wisniewski, Droby, Chalutz & Eilam, 1995). In our study, the Richard's solution contained 150 mM sucrose and this could explain the moderate growth inhibition we observed.

Several studies have attempted to determine the location of Ca^{2+} within the fungal cell. Precipitates of Ca-antimonate were found within nearly all cell organelles, including mitochondria, vacuoles, golgi-like bodies, nuclei and endoplasmic reticulum (Morales & Ruiz-Herrera, 1989). There is ample evidence to conclude that Ca^{2+} has an important role in tip growth of fungal hyphae, and that Ca^{2+} uptake is localized in the apex of the tip (Gooday & Gow, 1990; Schmid & Harold, 1988). Normally, an electrochemical gradient allows passive diffusion of Ca^{2+} ions into the cell through protein channels, but the low cytoplasmic Ca^{2+} concentration is mainly maintained through the activity of Ca/ATPase pumps (Rosen, 1982). Kaile, Pitt and Khun (1992) showed that *B. cinerea* hyphae accumulated Ca^{2+} when incubated with high Ca^{2+} concentrations up to 29.4 g l^{-1} $CaCl_2$ and, consequently, mycelial viability was reduced. P and K^+ are reported to be the most abundant ions in fungal hyphae (Lilly, 1965) and our results support those observations. These ions were also the most abundant in the culture medium that we used.

P plays an important role in metabolism and energy

Table 5
Calcium chloride effect on solubility and proportion of cell wall neutral sugars of *B. cinerea* hyphae[a]

	$CaCl_2$ in culture media (g l^{-1})					
	0	1	2	4	8	16
Neutral sugar hydrolysed in HCl 6N[b]	17.20 a	11.94 a	10.60 a	8.07 a	13.30 a	10.37 a
% Mannose	8.33 d	9.00 cd	10.28 bcd	10.22 bcd	22.55 abc	12.61 ab
% Glucose	81.70 a	80.56 ab	78.20 abc	76.44 abc	64.34 c	71.32 c
% Galactose	9.98 b	10.45 b	11.52 b	13.35 ab	13.09 a	16.06 a

[a] Duncan's multiple range test was used for statistical analysis; means within rows with the same letter are not significantly different; $P < 0.05$.
[b] expressed in mg 100 mg^{-1} dry wt hyphae.

transfer (Blumenthal, 1965), and is an integral component of DNA, RNA, phospholipids and coenzymes. We observed that when Ca^{2+} accumulated in the hyphae, P concentration also increased. This result was in agreement with the observation that phosphates facilitate Ca^{2+} accumulation in fungi (Callot, Moussain & Plassard, 1985). P can be part of the lipids present in the cell wall (Koulali, Fonvielle, Touze-Soulet, Benizri & Dargent, 1992) or attached to chitin and glycoprotein (Harold, 1962). K^+ ion absorption by the hyphae would use an electron-transport system (redox pump H^+/K^+) as suggested by Conway (Conway, 1953) and would regulate the cellular osmotic potential of the cell. This cation can bind to proteins within the cell and activate enzymes such as pyruvate kinase (Garraway & Evans, 1984). Mg^{2+} is essential to all fungi. It is a cofactor in enzymatic reactions, stabilizes the plasma membrane, and its uptake is ATP dependent. Fe is also essential to all fungi and is part of the structure of cytochrome and cytochrome oxidase. Only traces of Zn, Mo, Mn, Cu and S were detected.

During the infection process, the majority of Ca^{2+} is released from the host cell wall and Ca^{2+} concentration in the apparent free space (AFS) increases (Kaile et al., 1992). Previous work also showed that Ca^{2+}−infiltration of plant tissue can increase the Ca^{2+} content of plant cell walls 5 to 6 times (Abbott, Conway & Sams, 1989; Conway et al., 1988). As a consequence, the strengthening of the plant cell wall by Ca^{2+} binding the polygalacturonates decreased plant susceptibility to infection (Conway & Sams, 1983). Our results also suggest that this potentially high Ca^{2+} concentration released in the AFS of Ca^{2+}−treated tissue during the fungal attack can limit the infection process through a direct effect of the cation on *B. cinerea*.

The cell walls of the *B. cinerea* isolate used in this study were richer in neutral sugars (control cell wall content being 58%) than the 30% previously reported by Koulali et al. (1992). Ca^{2+} accumulation seemed to inhibit the synthesis of those polymers, possibly through an effect on enzymes such as β-glucan-synthases. Our isolate contained 15% glucosamine, the only aminosugar detected, which was twice that found by Koulali et al. (1992). When grown at 1 g l^{-1} $CaCl_2$, glucosamine concentration increased significantly. Ca^{2+} accumulation in the hyphae did not inhibit the enzymatic activities involved in chitin synthesis. Previous work has shown that chitin synthetase was activated by Ca^{2+} concentrations as low as 2–5 mM Ca^{2+} (Martinez-Cadena & Ruiz-Herrera, 1987).

The presence of uronic acids in the fungal cell wall has not always been acknowledged (Crook & Johnston, 1962), but recent studies showed that they are part of the fungal cell wall (Blake & Richards, 1970), and are present in *B. cinerea* cell walls (Koulali et al., 1992). Their low concentration in the cell wall could explain why the Ca^{2+} content in the control wall was so low. The ratio calculated {Ca equivalent/galacturonic acid equivalent} was 0.25 in the control wall and increased to 2.04 at 2 g l^{-1} $CaCl_2$. Ca^{2+} continued to be integrated into the cell wall which indicated that there were other binding sites for Ca^{2+}, perhaps the phosphates that accumulated in the cell wall. No significant difference in the total amount of cell wall compounds was found, mainly due to a high percent of variability in the data.

Fungal glucans are involved in surface recognition of susceptible hosts and in elicitation of the defense response of plants towards pathogens and play an important structural role (Ruiz-Herrera, 1992). The thickening of the fungal cell wall, a consequence of the high osmotic pressure developed when $CaCl_2$ concentrations increased could retard the elongation phase of the hyphae and result in a loss of cell wall elasticity. This effect of Ca^{2+} has already been shown in Ca-treated plant cells but to our knowledge, this phenomenon has not been reported in fungi. In addition, external Ca^{2+} can play an indirect role in fungal growth by altering internal Ca^{2+} which controls the cytoplasmic Ca^{2+} gradient, vesicle migration to the tip and the activity of fungal enzymes involved in cell wall expansion. One mechanism by which Ca^{2+} is known to reduce decay is by stabilizing the host cell wall and thus reducing maceration by cell wall degrading enzymes produced by fungal pathogens (Garraway & Evans, 1984). The results of this study suggest that the direct effect of Ca^{2+} on the fungal cell wall may also be a significant factor in host-pathogen interactions.

4. Experimental

4.1. Fungal material

Botrytis cinerea isolated from decayed apples (ATCC 90870, The American Type Culture Collection, Rockville, MD) was grown on potato carrot medium (PCM) and the resulting conidia transferred to potato dextrose agar (PDA). Conidial suspensions (2×10^4 spores ml^{-1}) were prepared from the PDA plates after 2 weeks of growth and used to inoculate 500 ml flasks containing 100 ml of Richard's soln with 50 g l^{-1} sucrose (Fahmy, 1923) as the carbon source. The pH of all media was adjusted to 4.2 and then $CaCl_2$ was added to the media at 0, 1, 2, 4, 8 or 16 g l^{-1}. The cultures were incubated at 22°C on a rotary shaker (50 rpm) under constant light. After 7 days, the mycelia were collected by centrifugation (8000*g*; 20 min; 4°C) and washed 3 times with ultrapure H_2O (quality >18 MW). The hyphae were dried at 70°C (until the dry wt was constant) and the dry wt determined. They were

then ashed at 500°C overnight and the resulting ash suspended in 2 N HCl. Mineral analysis was performed on 10 replicates for each $CaCl_2$ concentration by inductively coupled argon plasma atomic emission spectrometry (ICP).

4.2. Extraction and purification of the cell walls

Mycelia were suspended in 20 ml ultrapure H_2O and homogenized, first with a polytron (5 min; 4°C), and then a tissue grinder (at 4°C) until the extraction was complete (confirmed by light microscopy observation). The mixture was then centrifuged at 4°C with decreasing speed (1000, 800, 500 and 2000*g*) to remove the cytoplasmic contaminants from the cell walls (Koulali et al., 1992). As an indicator of purity, the absorbance of the washing solutions was measured at 260 nm (Work, 1971). Finally, the cell walls were collected by centrifugation at 2000*g* and freeze-dried.

4.3. Biochemical techniques for cell walls

4.3.1. Mineral composition

Three 15 mg dry wt fractions of freeze-dried cell walls from each treatment were analyzed for mineral composition as described above for the hyphae.

4.3.2. Protein determination

Proteins were extracted from the dried material after solubilization with 1 N NaOH for 12 h at room temperature (10 mg cell wall ml^{-1}), followed by 1 h in a boiling water-bath (Koulali et al., 1992). The protein content was determined according to the method of Lowry, Rosenbrough, Farr and Randall (1951). Three replicates of each treatment were analyzed.

4.3.3. Neutral sugar and uronic acid determination

Cell walls (10 mg) were dissolved in 2 ml of conc H_2SO_4 while stirring at 4°C. Ultrapure H_2O (500 ml) was added dropwise and after 5 min, 500 ml of ultrapure H_2O were added until the dissolution was complete (El Rayah Ahmed & Labavitch, 1977). The final volume was adjusted to 10 ml with ultrapure H_2O and the total neutral sugar (Mokrasch, 1954) and uronic acid (Blumenkrantz & Asboe-Hansen, 1973) contents were determined. Five replicates were analyzed.

4.3.4. Aminosugar determination

The aminosugars were extracted with 6 N HCl (3 mg cell walls ml^{-1}) at 100°C for 6 h (Koulali et al., 1992). The hydrolysates were then dried under an air stream, the residues washed 3 times with ultrapure H_2O, and suspended in 2 ml ultrapure H_2O. The glucosamine content was determined following the method of Sawicki and Johnson (Sawicki & Johnson, 1966), using 2–5 dimethyltetrahydrofuran and *p*-dimethylaminobenzaldehyde. Aminosugar content was confirmed by GC-MS analysis which was performed following the method described by Blakeney, Harris, Henry and Stone (1983). Three replicates of each treatment were analyzed.

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